bjh research paper

Frequency and characterization of known and novel *RHD* variant alleles in 37 782 Dutch D-negative pregnant women

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Received 7 October 2015; accepted for publication 14 December 2015

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Summary

To guide anti-D prophylaxis, Dutch D- pregnant women are offered a quantitative fetal-*RHD*-genotyping assay to determine the *RHD* status of their fetus. This allowed us to determine the frequency of different maternal *RHD* variants in 37 782 serologically D- pregnant women. A variant allele is present in at least 0·96% of Dutch D- pregnant women The D- serology could be confirmed after further serological testing in only 54% of these women, which emphasizes the potential relevance of genotyping of blood donors. 43 different *RHD* variant alleles were detected, including 15 novel alleles (11 null-, 2 partial D- and 2 DEL-alleles). Of those novel null alleles, one allele contained a single missense mutation (*RHD*443C>G*) and one allele had a single amino acid deletion (*RHD*424_426del*). The D- phenotype was confirmed by transduction of human D- erythroblasts, consolidating that, for the first time, a single amino acid change or deletion causes the D- phenotype. Transduction also confirmed the phenotypes for the two new variant DEL-alleles (*RHD*721A>C* and *RHD*884T>C)* and the novel partial *RHD*492C>A* allele. Notably, in three additional cases the DEL phenotype was observed but sequencing of the coding sequence, flanking introns and promoter region revealed an apparently wild-type *RHD* allele without mutations.

Keywords: Rh blood group, *RHD* variant alleles, D- phenotype, blood group genotyping.

The D antigen of the Rh blood group system is one of the most immunogenic and complex blood group antigens (Westhoff, 2007a; Daniels & Reid, 2010). Most D- individuals lack the complete RhD protein (Colin *et al*, 1991; Wagner & Flegel, 2000), which underlies its high immunogenicity. Anti-D can cause severe haemolytic transfusion reactions and/or severe haemolytic disease of the fetus and newborn. To prevent anti-D formation in D- individuals, compatible D- red blood cells (RBCs) are transfused and anti-D prophylaxis is administrated to D- pregnant women (de Haas *et al*, 2015).

The Rh locus is highly polymorphic and many *RHD* variant alleles have been described (Flegel, 2011). One group of *RHD* variant alleles, the *RHD* hybrid alleles, arose due to genetic recombination between the *RHD* gene and the adjacently located *RHCE* gene. The second group of *RHD* variant alleles carry one or multiple mutations in the *RHD* gene. The genetic variation of the *RHD* alleles has different effects on the level of expression of RhD protein and the number of expressed RhD epitopes. To date, more than 60 so-called D-null alleles have been described that cause the D- phenotype due to nonsense mutations, frame shift mutations, splice site mutations or to large hybrid alleles (http://www.uni-ulm.de/~fwagner/RH/RB2/P_RHDDnegative.htm). The D-null alleles *RHD*03N.01* and *RHD*Ψ* occur frequently in the D- African population (Flegel, 2011). *RHD* positive haplotypes are rare in D- Caucasians (Wagner *et al*, 2001; Flegel *et al*, 2005; Chou & Westhoff, 2010). Individuals with a weak D phenotype express the RhD protein in low quantities, which is most often caused by mutations in the transmembrane regions of the RhD protein (Daniels, 2013a). Individuals with DEL-allele expression have an even lower amount of the RhD protein on their RBC membrane, which can only be detected with the very sensitive adsorption-elution technique (Okubo *et al*, 1984). DEL expression is most often caused by missense mutations causing aberrant splice sites (Reid *et al*, 2012). Partial D expression, in which one or more D epitopes are lacking, is most often caused by hybrid alleles or due to mutations in the extracellular parts of the RhD

First published online 27 March 2016
doi: 10.1111/bjh.13960

protein. Some partial *RHD* variant alleles cause partial and weakened expression of the D antigen (Westhoff, 2007b). The distinction between the different variant alleles is of importance, given that it is unlikely that individuals with weak D or DEL expression produce allo-anti-D, in contrast to individuals with partial D expression who are at risk of D immunization (Daniels, 2013a; Sandler *et al*, 2015).

The aim of our study was to determine the frequency of (known and novel) *RHD* variant alleles in the serologically D- Dutch population. Since July 2011, Dutch D- pregnant women have been offered a quantitative fetal-*RHD*-genotyping assay to guide anti-D prophylaxis. This quantitative fetal-*RHD*-genotyping assay is performed with cell-free DNA isolated from maternal plasma, which contains DNA of the fetus (Scheffer *et al*, 2011; van der Schoot *et al*, 2013). However, the large majority of cell free DNA is of maternal origin and, therefore, if a maternal *RHD* allele is present it will be recognized because it results in much stronger signals in the quantitative polymerase chain reaction (PCR) assay than expected to arise from fetal DNA. In this paper we present the frequency of *RHD* variant alleles and serological and genetic follow up of cases identified among 37 782 screened Dutch D- pregnant women.

Material and methods

*Samples and fetal-*RHD*-genotyping assay*

Between July 2011 and December 2012, 37 782 Dutch serologically D- pregnant women (determined using two anti-D reagents) were tested in the 27th week of pregnancy for the presence of a D+ fetus using a quantitative fetal-*RHD*-genotyping assay. DNA was isolated from 1 ml of maternal plasma using a DNA isolation kit (DNA and Viral NA Large Volume Kit; Roche Holding AG, Basel, Switzerland) on a MagNa Pure 96 Instrument (Roche) according to the manufacturer's protocol. The quantitative fetal-*RHD*-genotyping assay has been described previously (Scheffer *et al*, 2011) and consists of a multiplexed TaqMan test, one targeting *RHD* exon 5 and one targeting *RHD* exon 7, performed in triplicate. When at least two of the three Ct values of both assays were below 32, a maternal variant allele was suspected. When at least two of the three Ct values of exon 7 were below 32, but Ct values of exon 5 were either negative or above 32, a *RHD*Ψ* or *RHD*06* maternal variant allele was suspected. In these cases additional genotyping and extended serology was performed to determine whether and which variant allele was present. Of note, the relatively frequently occurring null allele *RHD*03N.01* (Daniels *et al*, 1998) is not amplified and thus not detected in this fetal-*RHD*-genotyping assay.

Serology

All samples were subjected to column testing on either the Ortho Biovue Inova system (Ortho, Raritan, NJ, USA) with an ABO D card containing anti-D monoclonal antibodies (MoAbs) D7B8, or the Biorad/Diamed Diana system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with an ABO DVI- card, containing anti-D MoAbs LHM59/20 (LDM3) and 175-2. Plasma of all women with a negative result in this serological assay were tested in the fetal-*RHD*-genotyping assay. A second comprehensive serological assay was performed when a maternal variant allele was suspected. The samples were tested with three monoclonal blend reagents (IgM clone TH28 and IgG clone MS26) (Sanquin Reagents, Amsterdam, The Netherlands and Immucor, Norcross, GA, USA) and IgM clone D7B8, IgG clone H112196 and IgG clone LORIFA (Ortho), and one IgG clone 5C8, a polyclonal IgG reagent (Bio-Rad Laboratories) in an immediate spin at room temperature, in a spin proceeded by 15 min incubation at 37°C and/or by indirect antiglobulin test. If, following this second test, the D- phenotype was suspected and the presence of the *RHD*Ψ* allele was excluded, absorption-elution was performed using the Gamma® ELU-KIT® II (Immucor) as per manufacturer's protocol using a polyclonal anti-D (Bio-Rad Laboratories) to detect a DEL allele. If the second serological test detected a partial D variant other than the DVI variant, the D-epitope expression of the variant allele was determined using an in-house RhD typing kit consisting of eleven monoclonal IgG antibodies and an additional six MoAbs from the ALBAclone Advanced Partial RhD typing kit (ALBA Bioscience, Edinburgh, UK). These MoAbs were tested in anti IgG + anti C3d gel columns (Bio-Rad laboratories).

RH-MLPA

Maternal DNA was isolated from white blood cells using a DNA extraction kit (QIAamp DNA Blood Mini Kit, Qiagen Benelux, Venlo, The Netherlands). To determine *RHD* copy number and the presence of *RHD* variant alleles, DNA samples were analysed with the *RH*-Multiplex Ligation-dependent Probe Amplification (*RH*-MLPA) assay (mix p401-A1, p402-A1 and p403-A1, MRC-Holland, Amsterdam, The Netherlands) (Haer-Wigman *et al*, 2013). In some cases an *RHCE* MLPA was performed using *RHCE*-specific probes to determine the copies of *RHCE* exons 1, 3, 4, 5, 6, 7, 9 and 10 (probes listed in Table SI). One case was tested with seven new MLPA probe combinations (targeting c.-698T, c.123A, c.149-4875A, c.149-882G, c.244T, c.335 + 2838C and c.1112G of *RHD* and *RHCE*) that were developed to determine the combined *RHD* and *RHCE* copy numbers of the 5′UTR, exon 1, 2 and 8 and intron 1 and 2 (Table SI).

The MLPA reaction was performed according to the manufacturer's protocol on a Veriti Thermocycler (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). A mixture of 1·0 μl MLPA sample, 8·5 μl Hi-Di™ Formamide (Applied Biosystems) and 0·5 μl GeneScan™ 500-Liz® Size Standard (Applied Biosystems) was analysed on a 3130 Genetic Analyser (Applied Biosystems). Data analysis was

performed using Genemarker software version 1.85 (Softgenetics, State College, PA, USA).

DNA sequencing

When indicated, all exons and intron boundaries of *RHD* were sequenced and/or the promoter region of *RHD* was sequenced (hg19, chr.1:g.25597899_25598887; primer sequences are shown in Supplementary Table SI or as published previously (Haer-Wigman *et al*, 2013). The PCR was performed on a Veriti thermocycler in a total volume of 20 μl, containing 50–100 ng DNA, 10 μl of 2× GeneAmp Fast PCR Master Mix (Applied Biosystems), 0·5 μmol/l forward and reverse primer. PCR conditions were: 10 s at 95°C, 35 cycles of 10 s at 95°C and a specific annealing/elongation temperature and time for each primer set ranging from 62 to 70°C, followed by 1 min at 72°C. PCR products were purified using ExoSAP-IT (GE Healthcare, Eindhoven, The Netherlands), according to the manufacturer's protocol. PCR products were sequenced with ABI BigDye Terminator v3.1 kit on an ABI 3130XL sequencer (Applied Biosystems).

Heterologous transfection system

The *RHD* coding sequence flanked by a BamH1 and Not1 digestion site was ordered from Invitrogen (Breda, The Netherlands) and cloned into a lentiviral vector containing IRES-GFP for bicistronic gene expression driven under the EF1α promoter. The c.424-426del, c.443G, c.492A, c.721C and c.1154C mutations were mutated into the wild-type *RHD* construct using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, Amstelveen, The Netherlands) according to the manufacturer's protocol (Table SI). Lentivirus was produced by transfecting 293T cells with helper plasmids using the Calcium Phosphate method (Sambrook & Russel, 2001). The supernatant containing the virus particles was then harvested for 3 days and concentrated through ultracentrifugation. Erythroblast from five different D- (ccddee) donors were cultured from peripheral blood mononuclear cells according to the protocol described by van den Akker *et al* (2010) and kept in expansion medium for 2–3 d. Erythroblasts were then lentivirally transduced with the *RHD* wild-type construct or the different variant constructs. After 48-h transduction, cells were transferred into StemSpan medium (Stem Cell technologies, Grenoble, France) supplemented with stem cell factor (SCF; supernatant equivalent to 100 ng/ml), erythropoietin (10 μ/ml, ProSpec; East Brunswick, NJ, USA), holotransferrin (0·5 mg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) and insulin (10 mg/ml; Sigma-Aldrich) in order to allow cell differentiation (van den Akker *et al*, 2010).

Cells were harvested within 5 days of differentiation and screened for D-expression by flow cytometry using six human monoclonal IgG anti-D of the ALBAclone Advanced partial RhD typing kit (ALBA Bioscience), namely LHM169/81, LHM76/59, LHM76/55, LHM169/80, LHM57/17 and LHM76/58. Data analysis was performed with FlowJo Version 8 software (TreeStar, Ashland, OR, USA).

To measure mRNA levels of the transduced variants, RNA was isolated from 1×10^6 differentiated erythroblasts using TRIzol (Life Technologies, Paisley, UK) (Chomczynski & Sacchi, 1987) and 1 μg of RNA was used to synthesize cDNA using random hexamers (Invitrogen). Real-time quantitative reverse transcription PCRs (RQ-PCRs) were performed using the reporter dye SYBR-green (Sybrgreen Mastermix, Applied Biosystems) on a StepOnePlus (Applied Biosystems) with *RHD*-specific primers as listed in Table SI.

Results

A variant RHD *allele is present in 0·96% of Dutch D- pregnant women*

Between 2011 and 2012, the fetal-*RHD*-genotyping assay, to determine whether anti-D prophylaxis is indicated, was performed on a total of 37 782 D- pregnant women. In 493 women (1·3%) a maternal variant allele was suspected based on Ct-values and genetic follow-up was performed in 309 of these cases (Table SII). Genetic follow-up was not performed in 184 cases due to (i) missing samples ($n = 31$); (ii) Ct-values of around 31–32 ($n = 83$) and analysis in cases with stored DNA had shown that this is virtually always due to high fetal DNA levels and (iii) PCR results pointed to the presence of either *RHD*Ψ* or *RHD*06* allele, as was shown in the first series of 159 cases with similar Ct values for exon 5 and exon 7 (Table I).

In 39 (12·6%) of the 309 evaluated D- pregnant women in whom genetic follow-up was performed, the *RHD* negativity was based on complete deletion of the *RHD* gene because the homozygous presence of the *RHD*01N.01* allele was confirmed. In these cases the obtained Ct-values arose from high fetal *RHD*-DNA concentrations.

In the remaining 270 women a variant allele was identified (Table I). The distribution of *RHD* alleles in these cases was used to calculate the distribution of *RHD* alleles in cases without follow-up (Table SII). We estimate that 0·96% (95% confidence interval [CI] 0·86–1·06%) of the Dutch serologically D- pregnant women had a variant allele containing *RHD* exon 5 and/or 7. The most frequently detected variant allele is the *RHD*Ψ* allele, which was present in 47% of the women carrying a variant *RHD* allele.

Pregnant D- women determined with standard serology carried at least 43 different variant alleles

In 218 of the 270 analysed cases carrying variant alleles and on whom genotyping were performed, the *RH*-MLPA genotyping assay directly identified a specific known *RHD* variant allele (listed in Table I). In the remaining 52 cases (as indicated in Table I) additional genotyping was performed,

Table I. *RHD* variant alleles detected in 270 women determined D- with standard serology.

RhD phenotype	*RHD* allele 1	*RHD* allele 2	MLPA	Number of cases positive for genotype
D-	*RHD*Ψ*	*RHD*01N.01*	c	98 (2)
	*RHD*Ψ*	*RHD*01N.03*	c	1
	*RHD*Ψ*	*RHD*03N.01*	c	13
	*RHD*Ψ*	*RHD*Ψ*	c	6
	*RHD*660delG*	*RHD*01N.01*	n	1
	*RHD*922G>T*	*RHD*01N.01*	n	2
	*RHD*952C>T*	*RHD*01N.01*	n	3
	*RHD*DEL5*†	*RHD*01N.01*	n	1
	*RHD*DEL8*†	*RHD*01N.01*	n	3 (1)
	*RHD*DEL9*†	*RHD*01N.01*	n	1
	RHD(2-10)*§	*RHD*01N.03*	i	1
	*RHD*124_125del*§	*RHD*01N.01*	n	1
	*RHD*335G>T*§	*RHD*01N.01*	n	1
	*RHD*424_426del*§	*RHD*01N.01*	n	1
	*RHD*443C>G*§	*RHD*01N.01*	n	1
	RHD[361T>A; 380T>C; 383A>G; 455A>C; 602C>G; 667T>G; 819G>A]*§	*RHD*01N.01*	i	1
	RHD[634 + 1T;1136T]*§	*RHD*01N.01*	n	1
	*RHD*1073 + 1G>T*§	*RHD*01N.01*	n	1
	*RHD*1074-1G>A*§	*RHD*01N.01*	n	3
	*RHD*1084C>T*§	*RHD*01N.01*	n	1
	*RHD*1174del*§	*RHD*01N.01*	n	1
Partial RhD	*RHD*05.07*	*RHD*01N.01*	c	1
	*RHD*06.01*	*RHD*01N.01*	c	6
	*RHD*06.02*	*RHD*01N.01*	c	40
	*RHD*06.02*	*RHD*03N.01*	c	1
	*RHD*10.02*	*RHD*01N.01*	i	2
	*RHD*11*	*RHD*01N.01*	c	9 (1)
	*RHD*15*	*RHD*01N.01*	c	12 (2)
	*RHD*17.02*	*RHD*01N.01*	c	11 (4)
	RHD[178C; 689T]*§	*RHD*10.01*	i	1
	*RHD*492C>A*§	*RHD*01N.01*	c	1
DEL	*RHD*01EL.01*	*RHD*01N.01*	c	9
	*RHD*01N.22*‡	*RHD*01N.01*	n	1
	RHD[602C>G; 667T>G; 819G>A; 919G>A]*	*RHD*01N.01*	i	2 (1)
	RHD[602C>G; 667T>G; 819G>A; 919G>A]*	*RHD*[602C>G; 667T>G; 819G>A; 919G>A]*	i	1
	*RHD*93_94insT*	*RHD*01N.01*	n	6
	RHD(1-9)*	*RHD*01N.01*	i	2
	RHD(1-9)*	*RHD*Ψ*	i	1
	*RHD*1252_1253insT*	*RHD*01N.01*	n	1
	*RHD*721A>C*§	*RHD*01N.01*	n	7
	*RHD*884T>C*§	*RHD*01N.01*	n	1
	*RHD*01*	*RHD*01N.01*	n	3
Weak RhD	*RHD*01W.01*	*RHD*01N.01*	c	3
	*RHD*01W.02*	*RHD*01N.01*	c	3
	*RHD*01W.05*	*RHD*01N.01*	c	2
	*RHD*01W.22*	*RHD*01N.01*	n	1
	*RHD*01W.38*	*RHD*01N.01*	n	1

Multiplex Ligation-dependent Probe Amplification (MLPA) result: c, conclusive; i, inconclusive; n, seemingly normal *RHD* gene. All *RHD* exons were sequenced if MLPA i or n.

(..) Cases where no extended serology could be performed.

†The literature has described that the *RHD*DEL5*, *RHD*DEL8* and *RHD*DEL9* alleles cause the DEL phenotype (Singleton *et al*, *2001*; Flegel *et al*, 2009; Reid *et al*, 2012); however, we detected a D- phenotype.

‡The literature indicates that *RHD*01N.22* allele causes D- phenotype (Reid *et al*, 2012); however, we detected a DEL phenotype.

§Novel variant allele.

because the *RH*-MLPA typing results were inconclusive (n = 11) or indicated the presence of a normal *RHD* gene (n = 41). Sequencing of all *RHD* exons revealed the presence of previously described *RHD* variant alleles in 26 of these 52 cases. In the other 26 cases, 15 novel alleles were recognized (listed in Table III), which were detected in single cases, except for the *RHD*721A>C* allele, detected in seven cases, and the *RHD*1074-1G>A* allele, detected in three cases (Table I). In one case the novel *RHD-RHCE-RHD* allele could only be characterized by developing an MLPA containing seven new probes. In combination with the Ccddee phenotype and the results of the *RHCE*-MLPA, it was concluded (as explained in Fig S1) that this case most likely carries the known *RHD*01N.03* allele (described to be associated with RhCe expression) and a novel allele in which *RHD* exon 1 is deleted (*RHD*(2-10)*). In one other case with a partial D expression in which the new allele could not be unambiguously be proven, we assume that the known *RHD*10.01* and a novel *RHD*[178A>C; 689G>T]* allele are present instead of two novel alleles (*RHD*689C>T* and *RHD*[178A>C; 689G>T; 1136C>T]*). Remarkably, in three cases, all with the DEL phenotype and normal RhCE expression (RhCcee phenotype), an apparently normal wild-type *RHD* allele without mutations in the coding sequence, flanking introns and promoter region, was detected. In summary, a total of 43 different *RHD* variant alleles, including 15 novel *RHD* variant alleles, were identified in the 270 cases (Table I).

Phenotype analysis of the fifteen novel variants using serology

Red blood cells from 259 cases (including all cases with novel variant alleles) out of the 270 cases with a variant *RHD* allele were available for additional serological analysis, including adsorption-elution. The initial D- phenotype was confirmed in 139 (54%) cases. In 33 (13%) cases a DEL phenotype, in 77 (30%) cases a partial D and in ten (4%) cases a weak D phenotype was determined (Table I). The D- phenotype was confirmed for the novel alleles: *RHD*1084C>T*, *RHD*124_125del* and *RHD*1174del* (*RHD*(2-10)*) (*RHD*335G>T, RHD*[634+1G>T, 1136C>T], RHD*1073+1G>T* and *RHD*1074-1G>A*) and the novel variant composed of the known *RHD*03.03* and *RHD*09.03.01* (Table III).

The novel variants *RHD*884T>C* and *RHD*721A>C* caused the DEL phenotype (Table II). The *RHD*492C>A* allele was serologically determined to lead to partial D expression, as epitope 5 (rD7C2) and epitope 8 (HIMA-36) were absent, whereas all other evaluated epitopes (including epitope 8 tested with LHM76/58) were detected (Table II). Several anti-D MoAbs were only positive in the indirect agglutination test indicating that this variant allele has next to partial D also weakened D expression (data not shown). The RBC expressing the novel *RHD*[178A>C; 689G>T]* allele next to the known *RHD*10.01* showed loss of epitopes 1, 5 and 8. However, as this corresponds to the epitope pattern for the *RHD*10.01* allele (Wagner *et al*, 2002), the exact phenotype of the new allele could not be determined (Table II).

RhD epitope expression of the variants based on erythroblast expression system. Of the novel variant alleles the most surprising were the two variant alleles that caused the D- phenotype based on mutations that resulted in a single amino acid substitution or a single amino acid deletion in transmembrane regions of the RhD protein (Fig S2): the *RHD*443C>G* (encoding p.Thr148Arg) and *RHD*424_426del* (encoding p.Met142del) alleles (Table III). We therefore applied our novel expression system, using transduced donor-isolated ccddee erythroblasts to confirm that the detected mutations were indeed solely responsible for the loss of expression. In addition, two other novel variant alleles, the partial RHD allele *RHD*492C>A* and the DEL allele *RHD*721A>C* were tested in this system. As a sensitivity control, and as a control for our expression system, the *RHD*01W.02* and *RHD*01* were also transduced (Fig 1). *RHD* transcript levels, demonstrated that *RHD* mRNA levels did not significantly differ between *RHD*01* and the *RHD* variants, indicating that the loss of expression was caused by the mutation and not by differences in transduction efficiency or RNA stability (Fig 2).

The erythroblast expression system confirmed the serological findings: the *RHD*443C>G* and the *RHD*424_426del* transcripts did not give rise to expression of any RhD epitope in erythroblasts (Table II and Fig 1). The transduced erythroblasts confirmed the partial D phenotype of the *RHD*492C>A* allele, since LHM59/19 (targeting epitope 8.2) and RD7C2 (epitope 5) were completely negative and was not suggestive of severely weakened expression (Fig 2). The *RHD*721A>C* allele (encoding p.Thr241Pro) was serologically determined to cause the DEL phenotype and this was confirmed in erythroblast expression system, even at lower expression levels when compared to the *RHD*01W.02* allele (Fig 1). Furthermore, only a few anti-D, LHM76/55 and LHM169/80 (targeting epitope 3 and 6/7 respectively) were detected as very weakly positive (Fig 1 and Table II).

Discussion

In the present study we determined that a variant *RHD* allele containing *RHD* exon 5 and/or 7 is present in ~0·96% of the Dutch D- pregnant women. Genetic follow-up determined that almost half of the women with a variant allele carried the *RHD*Ψ* variant allele and about 16% carried one of the *RHD*06* variants. All other detected *RHD* variants are rare and in total we identified 43 different variant alleles, including 15 novel *RHD* variant alleles. Extensive serological follow-up confirmed the D- phenotype in 54% of the women, but partial D expression was found in 29%, DEL expression in 14% and weak D expression in 4% of the women. In three cases we detected an apparently normal wild-type *RHD* allele

Table II. D-epitope expression in the *RHD*443G*, *RHD*492A*, *RHD*[178C;689T]*, *RHD10.01*, *RHD*721A*, *RHD*01W.02* and *RHD*01* alleles.

Monoclonal antibody	RhD epitope	*RHD*01*		*RHD*01W.02*		*RHD*424_426del*		*RHD*443C>G*		*RHD*492C>A*		*RHD*721A>C*		*RHD*[178A>C;689G>T]* and *RHD*10·01*	*RHD*10·01*
		IAT	FACS	IAT	FACS	IAT	FACS	IAT	FACS	IAT	FACS	IAT	FACS	IAT	IAT
LHM70	1	3				–		–		+		–		–	–
LHM169/80	1	4	++		+	–	–	–	–	4	++	–	–	+	–
5C8	2	3				–		–		3		–		+	
LHM76/59	3		++		+	–	–	–	–		++	–	–		+
LHM76/55	3	4	++		+	–	–	–	–	3	+	+/–	+/–	+	+
AUB-2F7/Fiss	5					–		–				–			–
RD7C2	5	2				–		–		–		–		–	
LHM50/28	6/7	4				–		–		4		–	–	+	
LHM169/80	6/7	4	++		+	–	–	–	–	4	++	–	+/–	+	+
LHM57/17	6/7	4	++		+	–	–	–	–	4	++	–	–	+	
LOSI	6/7	4				–		–		4		+/–		+	
HIRO-5	6/7	4				–		–		4		–		+	
LHM76/58	8	4	++		+	–	–	–	–	4	++	–	–	+	+
HIMA-36	8	3				–		–		–		–	–	–	
LHM77/64	9		++		+	–		–		4		–	–	+	+
MS26	9					–		–		3		+/–		+	+
Blend anti-D (MS26+TM28)	6/7,9	4				–		–		3		–			
Polyclonal anti-D		3				–				3		–		+	

D-epitope expression as determined by serology and heterologous expression system for the novel variants *RHD*[178A>C;689G>T]*, *RHD*424_426del*, *RHD*443C>G*, *RHD*492C>A* and *RHD*721A>C*. As a sensitivity control, the well-known variants *RHD*01*, *RHD*10.01* and *RHD*01W.02* were also determined.
IAT, indirect antiglobulin test; FACS, fluorescence-activated cell sorting.

Table III. Fifteen novel variant alleles.

Allele	Nucleotide Changes†	Exon (Intron)	Protein change(s)‡	Initial serology	Extended serology	Adsorption-Elution	RhD phenotype	Linked *RHCE* genotype
*RHD*1084C>T*	c.1084C>T	8	p.Gln362Ter	Neg	Neg	Neg	D-	*RHCE*02*
*RHD*124_125del*	c.124_125delAA	1	p.Lys42 fs	Neg	Neg	Neg	D-	*RHCE*02*
*RHD*1174del*	c.1174delA	9	p.Ile392 fs	Neg	Neg	Neg	D-	*RHCE*02*
RHD(2-10)*	c.1-?_148 + ?del	1	p.0?	Neg	Neg	Neg	D-	*RHCE*01*
*RHD*335G>T*	c.335G>T	2	pSer112Ile r.spl?	Neg	Neg	Neg	D-	*RHCE*01*
RHD[634 + 1G>T; 1136T]*	c.[634 + 1G>T; 1136C>T]	(4), 8	r.spl?	Neg	Neg	Neg	D-	*RHCE*01*
*RHD*1073 + 1G>T*	c.1073 + 1G>T	(7)	r.spl?	Neg	Neg	Neg	D-	*RHCE*03*
*RHD*1074-1G>A*	c.1074-1G>A	(7)	r.spl?	Neg	Neg	Neg	D-	*RHCE*02*
RHD[361T>A; 380T>C; 383A>G; 455A>C; 602C>G; 667T>G; 819G>A]*	c.[361T>A; 380T>C; 383A>G; 455A>C; 602C>G; 667T>G; 819G>A]	3, 4, 5, 6	p.[Leu121Met; Val127Ala; Asp128Gly; Asn152Thr; Thr201Arg, Phe223Leu; Ala273Ala]	Neg	Neg	Neg	D-	*RHCE*01*
*RHD*443C>G*	c.443C>G	3	p.Thr148Arg	Neg	Neg	Neg	D-	*RHCE*02*
*RHD*424_426del*	c.424_426delATG	3	p.Met142del	Neg	Neg	Neg	D-	*RHCE*02*
*RHD*721A>C*	c.721A>C	5	p.Thr241Pro	Neg	Neg	Pos	D^{el}	*RHCE*01*
*RHD*884T>C*	c.884T>C	6	p.Met295Thr	Neg	Neg	Po	D^{el}	*RHCE*02*
RHD[178A>C; 689G>T]*	c.[178A>C; 689G>T]	2, 5	p.[Ile60Leu; Ser230Ile]	Neg	Partial§	-	Partial weak D	*RHCE*01*
*RHD*492C>A*	c.492C>A	4	p.Asp164Glu	Weak	Partial	-	Partial weak D	*RHCE*02*

†Position as counted from ATG translation start site; homo = homozygous, hetero = heterozygous.

‡Position as counted from Met translation start site.

§The case positive for this variant allele carried a second variant allele *RHD*10.01* that causes partial D expression, therefore we cannot exclude that this variant allele can also cause the D- phenotype.

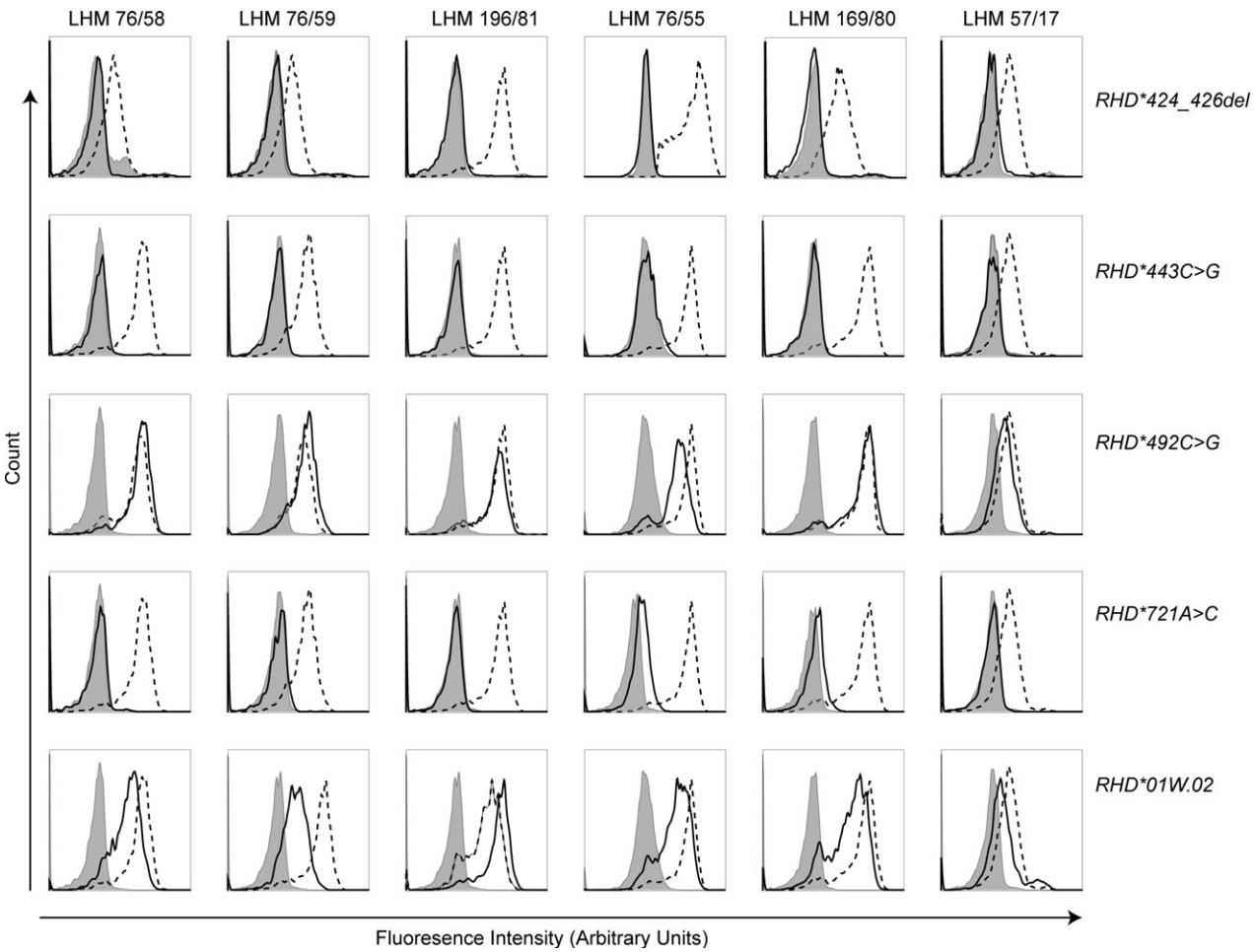

Fig 1. RhD expression levels of the *RHD*424_426del*, *RHD*443C>G*, *RHD*492C>A* and *RHD*721A>C* variant alleles in a heterologous expression assay. Overlay plots of the fluorescence intensity, representative for the RhD expression levels of RhD-negative erythroblasts transduced with constructs containing the *RHD*424_426del*, *RHD*443C>G*, *RHD*492C>A*, or *RHD*721A>C* and the well-described *RHD*01W.02* cDNA (black line). The wild-type *RHD*01* cDNA (black dashed line) was transduced for quantitative analysis of the expression levels. The *RHD*01W.02* sensitivity control showed weakened RhD expression levels compared to the *RHD*01* wild-type allele. The *RHD*424_426del*, *RHD*443C>G* alleles had completely no RhD expression, the *RHD*492C>A* allele had similar expression levels to the wild-type *RHD*01* but showed weakened expression of epitope 3 (LHM76/55). The *RHD*721A>C* showed very weak expression of epitope 3 (LHM76/55) and epitope 6/7 (LHM 169/80) weakened RhD expression, even weaker than the *RHD*01W.02* allele. Histograms are representative figures (n = 3).

yet a DEL phenotype was observed. Our analysis shows that in 0·22% (95% CI 0·17–0·26%) of cases (excluding the women carrying the *RHD*06* variants) serological typing incorrectly indicated RhD negativity. Conversely, *RH*-MLPA incorrectly predicted the D+ phenotype in 0·05% (95% CI 0·03–0·08%) of serologically D- women.

Here we describe 15 novel alleles (11 D-null, two partial-weak and two DEL alleles). The D- phenotype was determined for a novel allele with a nonsense mutation (*RHD*1084C>T*), two novel alleles with frame shift mutations (*RHD*124_125del* or *RHD*1174del*), four alleles with mutations that disrupt a splice site (*RHD*335G>T*, *RHD*[634+1G>T, 1136C>T]*, *RHD*1073+1G>T* and *RHD*1074-1G>A*) and one allele with the deletion of exon 1 (*RHD*(2-10)*). The presence of this last allele could not be unambiguously proven, because of the presence of another variant allele in this case.

The D- phenotype was also determined for a variant allele that contained mutations of both the *RHD*03.03* and *RHD*09.03* variant alleles (*RHD*[361T>A; 380T>C; 383A>G; 455A>C; 602C>G; 667T>G; 819G>A]*). This was unexpected because the *RHD*09.03* allele causes only a moderate weakening of the RhD expression and the *RHD*03.03* allele has not been associated with an effect on RhD expression levels, although the mutations are all found in putative transmembrane and intra-cellular parts of the RhD protein, which might offer some explanation for our finding.

Interestingly, both an allele with a single missense mutation *RHD*443C>G* (encoding p.Thr148Arg) and an allele with the deletion of a single amino acid *RHD*424_426del* (p.Met142del) cause the D- phenotype, which was confirmed in a heterologous expression study in D- erythroblasts. Both alleles have mutations in the fifth putative transmembrane

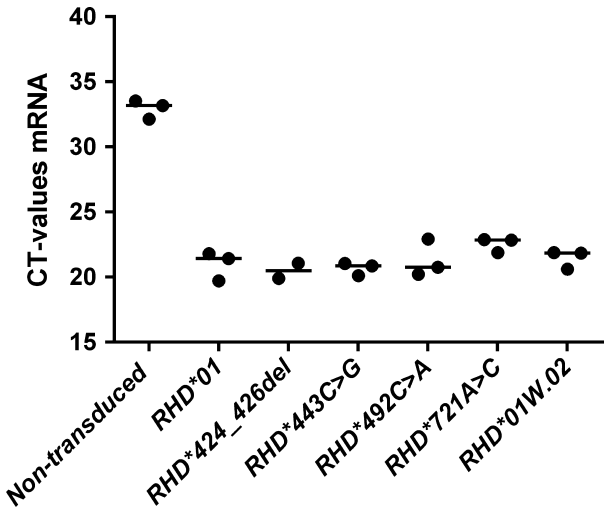

Fig 2. RhD-transcript levels of D- transduced erythroblasts with the novel variants: *RHD*424_426del*, *RHD*443C>G*, *RHD*492C>A* and *RHD*721A>C*. Transcript levels of the various *RHD* mRNA show no significant differences between the novel variants or our sensitivity controls (*RHD*01, RHD*01W.02*) indicating that the fluorescence-activated cell sorting results do not depend on different levels of viral transduction. Each dot represents one D-negative erythroblast donor.

region of the RhD protein. Other *RHD* variant alleles with mutations in this region, show only drastically diminished D expression, for instance the *RHD*01EL.07* (p.Ala137Glu) and *RHD*01EL.12* (p.Leu153Pro) alleles (Flegel *et al*, 2009; Li *et al*, 2009). The mutations present in the transmembrane region may either influence RhD binding to RhAG and thereby disrupt the RhAG-RhD oligomer stability, or disrupt correct protein folding (Callebaut *et al*, 2006).

The phenotype of the presumed novel partial *RHD* [178A>C;689G>T]* allele could not be unambiguously proven, because this allele was present next to the known *RHD*10.01* allele and the observed epitope pattern (loss of epitopes 1, 5 and 8 and weakened D expression of the other epitopes) was similar to that described for *RHD*10.01* (Wagner *et al*, 2002). The c.689G>T mutation is responsible for the partial weak D phenotype in the known *RHD*10.01 (RHD*[689G>T;1136C>T]*) allele, because the *RHD*10.00 (RHD*[1136C>T])* has normal RhD expression (Wagner & Flegel, 2002). This could mean that, for the case carrying the *RHD*10.01* allele and the *RHD*[178A>C;689G>T]*, the *RHD*10.01* allele alone is responsible for this partial weak D phenotype but it is possible that the novel *RHD* [178A>C;689G>T]* variant results in a similar phenotype as both variants carry the c.689G>T (p.Ser230Ile) mutation.

The novel variant allele *RHD*492C>A* (p.Asp164Glu) equally caused partial weak D expression on RBCs and, also in the heterologous expression system, epitope 5 and part of epitope 8 were absent.

The novel *RHD*721A>C* allele (p.Thr241Pro) and *RHD*884T>C* (p.Met295Thr) with a single missense mutation in the transmembrane region of the RhD protein causes the DEL phenotype. Interestingly, the *RHD*721A>C* allele was detected in seven cases whereas all other novel alleles, except the *RHD*1074-1G>A* allele, were detected in single cases. This allele was not detected in previous studies performed in Germany, Austria, Poland and Belgium, respectively (Flegel *et al*, 2009; Polin *et al*, 2009; Orzinska *et al*, 2013; Van Sandt *et al*, 2015, respectively, indicating that this allele is specific for the Dutch population. All women positive for the *RHD*721A>C* allele had Dutch surnames but we have no indication that these women are related.

The DEL and RhCcee phenotype was detected in three cases with a wild-type *RHD*01* allele and without any mutation in the intron boundaries or in the promoter region of the *RHD* gene. The RhCe expression was normal in these two cases. Flegel *et al* (2009) also described a single case without mutations in the *RHD* exons and intron boundaries with the DEL and a normal RhCe phenotype. Possibly, in these cases a deep intronic mutation is present or a gene that is required for membrane expression of the RhD protein is mutated.

Furthermore, it is important to note that for the variants *RHD*DEL5*, *RHD*DEL8*, *RHD*DEL9*, *RHD*01N.22*, the D- phenotype determined in this study deviated from previously reported DEL phenotypes (Singleton *et al*, 2001; Flegel *et al*, 2009; Reid *et al*, 2012). A D- phenotype for *RHD*DEL8* and *RHD*DEL9* has been described (Wagner *et al*, 2001) and for *RHD*DEL8*, Kormoczi *et al* (2005) described a partial D phenotype. Moreover, in agreement with our observed D- serology is the fact that alloimmunization has occurred in individuals carrying the *RHD*DEL8* (Kormoczi *et al*, 2005; Gardener *et al*, 2012) or the *RHD*DEL5* (Daniels & Reid, 2010) allele.

Three large studies have been performed (46 133 D- donors in Germany (Flegel *et al*, 2009), 31 200 D- donors in Poland (Orzinska *et al*, 2013) and 23 330 D- donors Austria (Polin *et al*, 2009)) to ascertain the presence of *RHD* variant alleles in D- donors, in which considerably lower percentages of D- donors carried *RHD* variant alleles, 0·21%, 0·20% and 0·40%, respectively. The African *RHD*Ψ* allele was observed at low frequency (0·03%) in the German donor population (Flegel *et al*, 2009) and not among the Austrian and Polish donors (Polin *et al*, 2009; Orzinska *et al*, 2013). Furthermore, in our study the *RHD*06* variant was tested as D- on purpose, while this was not the case in other studies. Even if the frequency of variant alleles in our study is recalculated excluding the *RHD*Ψ* and the *RHD*06* alleles, 0·32% (95% CI 0·26–0·37%) of the D- women carry an *RHD* variant: this is still slightly higher than the frequency in the two largest blood donor studies, possibly reflecting the multiracial origin of the Dutch pregnant population.

In conclusion, 0·96% of the Dutch D- pregnant women carry a D variant allele harbouring *RHD* exon 5 and/or exon 7. The large majority of pregnant women with a variant allele carry an *RH*-null allele or partial *RHD* allele and need administration of anti-D prophylaxis to prevent anti-D immunization. Genotyping of this group of women has the

limited advantage in that the woman with weak D type 1, 2 and 3 are recognized and can be regarded in the current and any subsequent pregnancy as D+. More importantly, our study emphasizes the relevance of genotyping of blood donors (Denomme, 2013; Sandler *et al*, 2015). Furthermore, this cohort of extensively typed D- women can be used to optimize *RHD* genotyping assays, as it is essential that the most frequently occurring D-null alleles are identified for correct prediction of the D phenotype via a genotyping assay (Gassner *et al*, 2005; Daniels, 2013b).

Author contributions

TS, BV, RB, PL and LHW performed the research. FT, BB, GC, RJ, BV and PL collected and registered all the samples. The data was analysed by TS, BV, LHW, CS and MH. The study was designed by CS and MH. TS, LHW and CS wrote the paper.

Conflicts of interest

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to *British Journal of Heamatology*.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Schematic representation of MLPA results in a single case with two *RHD* variant alleles.

Fig S2. Position of mutations of seven alleles in the RhD protein.

Table SI. Primer and probe list list.

Table SII. Calculation of number of cases with a *RHD* variant allele in the total cohort of 37 782 D- pregnant women.

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